# Soluble Suppressor Factors in Patients with Acquired Immune Deficiency Syndrome and Its Prodrome

## ELABORATION IN VITRO BY T LYMPHOCYTE-ADHERENT CELL INTERACTIONS

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ABSTRACT Supernatants from peripheral blood mononuclear cells obtained from certain patients with the acquired immune deficiency syndrome (AIDS) or its prodrome were capable of depressing spontaneous and pokeweed mitogen-driven B lymphocyte differentiation into plasmacytes, and the proliferative responses of T cells to specific antigen. These soluble suppressor factors (SSF) were present in uniquely high concentrations, with significant differences from healthy controls and from patients with various other conditions previously associated with factor-mediated immunosuppression. T cell-independent functions were not modified by SSF. Suppression was not genetically constrained, and did not appear to be mediated by cytotoxicity, prostaglandin, or alpha or gamma interferons.

SSF was a product of the interaction of T lymphocytes with adherent cells. T cells or T cell factors from AIDS patients, but not from normal controls, could collaborate with control adherent cells in the formation of SSF.

Restoration of DNA synthesis-independent differentiation of B lymphocytes into plasmacytes in SSF-treated cultures was realized by addition of reducing agents, such as 2-mercaptoethanol, on culture initiation. These data suggest inhibitory mechanisms possibly related to that of concanavalin A-induced soluble immune response suppression, and perhaps offer clues to clinically applicable substances which are potentially capable of mitigating such responses.

## INTRODUCTION

Acquired immune deficiency syndrome (AIDS)[1] is defined as a disease (opportunistic infection, or Kaposi's sarcoma in an individual < 60 yr of age) which is predictive of a defect in cell-mediated immunity and arises in a person without known predisposition for diminished resistance to that disease (1). The common denominator in patients with AIDS is a profound but selective abnormality of immunoregulation. Virtually all individuals show a deficit in cellular immune response (2, 3). Although isohemagglutinin titers, serum immunoglobulin (Ig) levels, and specific secondary antibody responses may be normal (2), primary antibody responses to antigens, such as keyhole limpet hemocyanin, are markedly depressed (4). A reduction in the relative number of the phenotypically characterized helper/inducer T cell subset, with a corresponding reversal of the normal ratio of helper/inducer to suppressor/cytotoxic T cell subpopulations in favor of suppression, has been documented (2, 3). Such a disturbance was also recorded in homosexual males with unexplained generalized lymphadenopathy (5) and, al-

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[1] *Abbreviations used in this paper:* AIDS, acquired immune deficiency syndrome; Con A, concanavalin A; E-rosette, sheep erythrocyte rosettes; EBV, Epstein-Barr virus; FCS, fetal calf serum; ID/thy, immunodeficiency and thymoma; 2-ME, 2-mercaptoethanol; PBMC, peripheral blood mononuclear cell; PFC, plaque-forming cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SIRS, soluble immune response suppressor; SSF, soluble suppressor factor.

beit to a less marked extent, in healthy homosexual males (6). These latter findings are of special concern as a spectrum of disorders that stem from persistent hyperplastic lymphadenopathy to AIDS is postulated to occur among susceptible groups (7).

The role of these T lymphocyte alterations in the pathogenesis of AIDS is unclear. Because of leukopenia and peripheral, as well as lymph node and splenic, lymphopenia in AIDS (2, 3, 8), the absolute number of phenotypic helper and suppressor T cells are typically decreased. Although the total number of circulating suppressor T lymphocytes in homosexual males with hyperplastic lymphadenopathy may be elevated, blastogenic responses to lectins are variable (5), and autoantibodies have been identified (7).

To elucidate the mechanisms underlying these aberrations, supernatant products released by lectin-free cultures of peripheral blood mononuclear cells (PBMC) derived from AIDS patients and male homosexuals with unexplained generalized lymphadenopathy were examined. These supernatants proved to contain potent inhibitors of spontaneous and pokeweed mitogen (PWM)-driven polyclonal Ig production, as well as blastogenic responses of T cells to specific antigen. Cell separation techniques demonstrated that these soluble suppressor factors (SSF) were the result of an interaction of T lymphocytes with adherent cells. Adherent cells from healthy controls could be induced to express SSF activity upon exposure to AIDS T cells or T cell products.

## METHODS

*Subjects.* The control group consisted of 22 individuals: 14 healthy heterosexuals, 10 male and 4 female, and 8 asymptomatic homosexual males, who lacked historical or physical evidence of lymphadenopathy, hepatosplenomegaly, recent fever, weight loss, or infection. The patient group included 16 homosexual males with unexplained generalized lymphadenopathy, defined as lymph nodes $> 1$ cm in diameter involving at least two extrainguinal chains for more than 3 mo., in the absence of current disease or drug use known to be associated with nodal enlargement. Two patients had palpable splenomegaly; 12 admitted contemporary symptoms of malaise, weight loss $\geq 10\%$ of ideal body weight, or intermittent temperature elevations between 37.5 and 38°C. One individual had clinically significant immune thrombocytopenic purpura. Five patients underwent diagnostic abdominal, axillary, or cervical lymph node biopsy, which revealed follicular hyperplasia. Four conform to the Centers for Disease Control definition of AIDS (1), with a prior record of opportunistic infection: one each with *Herpes simplex* encephalitis and *Pneumocystis carinii* pneumonia, and two with esophageal candidiasis. Individuals were free of disease and off all medication at the time blood specimens were obtained. The age range for controls was 22–36 yr, and for patients 25–45 yr.

One patient with immunodeficiency and thymoma (ID/thy-M.P.) was also included in this study. She had depressed serum Ig levels and elevated circulating suppressor T lymphocytes, as previously determined by phenotypic analysis with monoclonal antibodies and in a functional co-culture assay (9).

### *Techniques*

*Lymphocyte and monocyte separation and identification.* PBMC were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation ($d = 1.077$), and fractionated into T and non-T subpopulations by rosetting with neuraminidase-treated sheep erythrocytes (E-rosette) for 45 min at 4°C. In select experiments, monocytes were segregated from PBMC by incubating $5 \times 10^6$ cells/ml in polystyrene tissue culture flasks in culture medium (RPMI 1640 plus 10% heat-inactivated fetal calf serum [FCS]) for 16 h at 37°C, and was followed by rinsing of the flasks with culture medium to remove nonadherent cells. Cold phosphate-buffered saline was added, the flasks kept at 4°C for 1 h, and adherent cells subsequently harvested by vigorous pipetting. The nonadherent fraction was further depleted of monocytes by treatment with carbonyl iron, which was followed by removal of iron-laden cells by passage through a magnetic field (10). Using these procedures, T lymphocyte populations routinely contained >95% E-rosette$^+$ cells, <0.5% esterase$^+$ cells, and <2% surface Ig-positive cells by immunofluorescence with a rabbit anti-human Fab′ reagent. Adherent cells were 60% esterase$^+$, 10–15% E-rosette$^+$, and 80–85% stained with a murine monoclonal antibody, 63D3, which is reactive with human monocytes. T lymphocytes were quantitated by indirect immunofluorescence by using monospecific antibodies to mature peripheral T lymphocytes, and helper/inducer and suppressor/cytotoxic T cell subsets (Leu-4, Leu-3a, and Leu-2a, respectively, which were kindly provided by Dr. Robert Evans, Memorial-Sloan Kettering Cancer Center, New York), as previously described (2).

*Complement-dependent lysis of lymphocytes with monoclonal antibodies.* $1 \times 10^6$ E-rosette$^+$ lymphocytes were incubated with 0.2 ml of anti-Leu-3a or anti-Leu-2a at a 1:200 final dilution for 45 min at 25°C. Rabbit complement (Pel-Freez Biologicals, Rogers, AK) was added to the mixture at a final dilution of 1:10; the mixture was incubated for 1 h at 37°C with intermittent shaking and washed three times with phosphate-buffered saline; and then, the entire protocol repeated two more times.

*Production of soluble factors.* 2-ml aliquots of PBMC or discrete PBMC subsets were suspended in culture medium at $2.5 \times 10^6$ cells/ml and incubated in polyvinyl flat-bottomed macrowells at 37°C in an atmosphere of 5% humidified $CO_2$ for 48 h. Supernatants were recovered and pooled from appropriate wells, filtered through 0.45 $\mu$ membranes (Millipore Corp., Bedford, MA), and stored at −20°C. To prepare concanavalin A (Con A)-stimulated factors, 10 $\mu$g/ml of Con A was added upon initiation of cell cultures. A pulsing procedure, which involved replacement of Con A supernatants with fresh medium devoid of lectin at 16 h of culture and subsequent harvesting of supernatants after an additional 32-h incubation, proved sufficient to eliminate 90% of the mitogen (11).

*Induction of in vitro Ig synthesis.* In preliminary experiments, PBMC were isolated from healthy heterosexual adult volunteers of known HLA phenotype. Lymphocytes were typed for HLA-DR antigens by the microdroplet lymphocyte cytotoxicity test with a battery of highly selected typing sera. They were fractionated into E-rosette$^+$ and E-rosette$^-$ (B plus adherent cells) subsets. Varying numbers of T cells ($2.25–0.028 \times 10^5$) were added to a constant number of autologous non-T cells ($7.5 \times 10^4$) in 0.3 ml of culture medium, and incubated with several concentrations of PWM (1:100–1:450) in flat-bottomed wells of polyvinyl microculture plates at 37°C. On day 6, the contents of each well were collected and polyclonal Ig production assessed in the reverse plaque assay, as previously detailed (12). Conditions that yielded optimal plaque formation were standardized for three individuals, and their PBMC were employed as "indicator cells" in all subsequent experiments. Ig production is expressed as numbers of plaque-forming cells (PFC) as counted by direct visualization, with random checks for pseudoplaques (areas of hemolysis without a central lymphocyte) by using a dissecting microscope. Results are recorded as the mean number of PFC obtained from duplicate cultures.

In select assays, non-T or purified B cells from these donors were stimulated with Epstein-Barr virus (EBV). Cell-free supernatants of the cell line MCUV served as the source of infectious virus. 0.3 ml of this supernatant was added to $3 \times 10^6$ cells that were sedimented in round-bottomed culture tubes and then, rocked continuously for 1 h at 37°C. Cells were washed three times with phosphate-buffered saline and $1.5 \times 10^5$ were incubated in 0.3-ml culture medium in flat-bottomed microwells at 37°C. The contents of each well were harvested on day 8 and evaluated in the reverse plaque assay.

*Stimulation of PBMC with mitogen and antigen.* Indicator cultures for the assessment of the effect of cell supernatant on T lymphocyte blastogenesis were established. $2 \times 10^5$ PBMC obtained from healthy donors were added to round-bottomed wells of polyvinyl microtiter plates with 0.2 ml of culture medium in the presence or absence of phytohemagglutinin (PHA, 1:2,000 final dilution) or Con A (10 $\mu$g/ml) for 3 d. In other experiments, $3 \times 10^5$ donor cells were incubated in flat-bottomed microwells with 1:150 final dilution of PWM for 5 d. 16 h before culture termination, 1.0 $\mu$Ci of [$^3$H-*methyl*]thymidine (spec act 1.9 Ci/mM, Becton Dickinson Immunodiagnostics, Orangeburg, NY) was added to each well. Cells were harvested onto fiberglass filters and cell-associated radioactivity determined. The results are expressed either as the mean counts per minute of triplicate wells, or as a stimulation index, which was calculated by the formula: Stimulation index = mean cpm of mitogen-activated cultures/mean cpm of unstimulated cultures.

To evaluate the effect of supernatants on T cell mitogenesis induced by specific antigen, indicator cultures were prepared by using PBMC from donors sensitized to tetanus toxoid. Tetanus toxoid (40 $\mu$g/ml, 630 Lf/ml) was added to cultures of $2 \times 10^5$ PBMC/flat-bottomed microwell in 0.25 ml of RPMI 1640 supplemented with 20% autologous heat-inactivated sera. Cultures were incubated in the presence or absence of supernatant (1:5 final dilution, vol/vol) for 6 d, with addition of [$^3$H]thymidine 16 h before harvesting. The results are expressed as percent suppression of control cultures, which was calculated as: Percent suppression = 1 − ([mean cpm stimulated culture + supernatant] − [mean cpm unstimulated culture + supernatant])/([mean cpm stimulated culture − supernatant] − [mean cpm unstimulated culture − supernatant]).

*Assessment of natural killer cells activity.* PBMC from a normal donor were depleted of adherent cells by consecutive steps of plastic adherence and nylon wool passage as previously described (13), and $5 \times 10^5$ cells were incubated with $1 \times 10^4$ $^{51}$Cr-labeled (100 $\mu$Ci for 90 min) K562 erythroleukemia targets in round-bottomed microwells in 0.2-ml culture medium. After 4 h of incubation at 37°C, 100 $\mu$l of supernatant was removed from each well for counting in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, IL), and percent specific lysis calculated (13). Supernatants from control and AIDS individuals were added, at initiation of this assay, in final dilutions of 1:4 and 1:40.

*Treatment of indicator cultures with indomethacin and anti-interferons.* A stock solution of indomethacin (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 50 mg in 10-ml absolute ethanol; the dilutions were made in culture medium so that 25 $\mu$l, when added to indicator cultures, gave a range of concentration between 1.0 and 10 $\mu$g/ml (2.8–28 $\mu$M). Stock solutions of a rabbit anti-human gamma interferon (graciously provided by Dr. Marlyn Langford, Dept. of Microbiology, University of Texas Medical Branch, Galveston, TX), and of a sheep anti-human alpha interferon (Interferon Sciences, Inc., New Brunswick, NJ) were made so that 1 ml contained 1,000 units of activity. (1 U is defined as the amount of reagent required to neutralize 10 U of gamma or alpha interferon to 1 U.) These antisera were added to indicator cultures to give 1–10 U/microwell.

### *Statistical analysis*

The observed distributions for data from all subject groups, as well as transformations of each, were plotted and compared by using a *t* test. The results support univariate analysis by using nonparametric methods.

## RESULTS

*Effect of lectin-free and Con A-stimulated PBMC supernatants on PWM-driven Ig production.* Significant suppression of polyclonal Ig production in PWM-induced indicator cultures by supernatants from unstimulated PBMC of healthy heterosexual and homosexual individuals was noted at the lowest dilution of supernatant evaluated (1:3 vol/vol, Fig. 1). There was no significant difference in mean suppression between these two groups ($P = 0.22$, Table I). Dilution of those supernatants that showed initial inhibitory activity often revealed increased PFC in target cultures; this suggests that helper and suppressor factors were present. In contrast to the control groups, high titers for PFC suppression were realized on dilution of lectin-free supernatants of AIDS/male homosexual lymphadenopathy patients (Fig. 1). Enhancement of PFC was noted in only one instance. Two individuals, AIDS-Sel and AIDS-Ton, with generalized lymphadenopathy and low grade fevers but no prior history of neoplasia or opportunistic infection, showed almost complete abrogation of target PFC responses at a titer of 1:1,000.

As shown in Table I, Con A induction of PBMC from healthy heterosexuals yielded supernatant inhibitory factors different from controls at a 1:3 but not a 1:27 dilution. However, lectin-free supernatants from AIDS patients suppressed PFC at both concentrations. Although there was no difference in mean PFC inhibition between SSF-AIDS and SSF-Con A at a titer of 1:3 ($P = 0.30$), there was a highly significant difference at greater supernatant dilutions.

Induction and expression of suppressor activity was supported by RPMI 1640 supplemented with 20% pooled human AB sera as well as the 10% FCS employed in the above experiments. Serum-free medium, con-

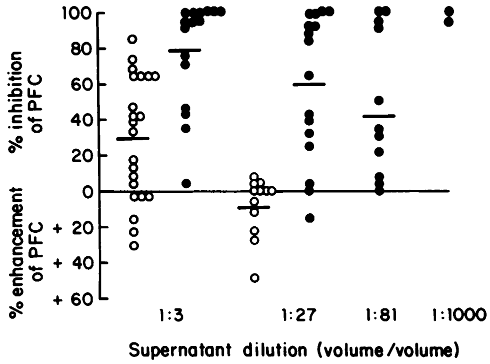


FIGURE 1 Effect of PBMC supernatants on PFC capacity of PWM-driven indicator cultures. The open circles (O) represent the mean of duplicate cultures incubated with the appropriate dilution of supernatant from PBMC of healthy heterosexual and homosexual controls. Only those supernatants showing ≥20% suppression at a 1:3 dilution were evaluated at 1:27. The filled circles (●) represent the mean of duplicate indicator cultures incubated with supernatant from PBMC of patients with AIDS or homosexual males with generalized lymphadenopathy. Only those supernatants eliciting ≥20% inhibition at a titer of 1:27 were evaluated at 1:81. Horizontal bars indicate mean suppression or enhancement for the appropriate group.

taining 1% bovine serum albumin, insulin, and transferrin, did not permit significant generation of SSF. As in preliminary studies, because different lots of FCS gave divergent titers of SSF activity, one lot was uniformly utilized in the preparation of culture medium.

Comparison of the inhibitory capacity of supernatants derived from heterosexual controls with those from persons with active bacterial or viral infection (lepromatous and tuberculous leprosy, subacute bacterial endocarditis), multiple myeloma, and the convalescent phase of EBV or cytomegalovirus mononucleosis, revealed PFC suppression greater than control levels only at a 1:3 dilution.

To assess whether secretion rather than Ig synthesis was modified by SSF-AIDS, plasmacytes were quantitated in the PWM-driven indicator cultures at the end of the 6-d incubation. Harvested cells were stained intracellularly by indirect immunofluorescence utilizing mouse monoclonal, isotype-specific anti-Fc reagents for IgG and IgM. Similar SSF-mediated inhibition was detected by enumeration of PFC or intracytoplasmic $Ig^+$ cells.

The possibility that SSF-AIDS, rather than being synthesized and released by cells in culture, reflected an inhibitory substance present in autologous sera and tightly adsorbed to cells was addressed in a time course experiment. Supernatants were harvested from cultures of AIDS PBMC from 2 to 72 h after culture initiation, and each aliquot was assessed in the PWM-driven PFC system. Supernatants collected at 2 h had no effect; SSF harvested at 12 h gave ~50% of the maximal inhibitory response, which was attained at 48–72 h.

To negate the fact that adsorption and consequent removal of available mitogen is responsible for PFC suppression, the effect of SSF on PFC capacity of normal PBMC cultured for 6 d without PWM was examined. Indicator cultures were established as described above, except that a donor who was known to yield high spontaneous plaques was selected. Unstimulated supernatant from AIDS-Ton and SSF-Con A from a normal control markedly depressed Ig production at a dilution (1:9) at which unstimulated control supernatant had no effect.

*Effect of SSF on cell viability and lymphocyte blas-*

TABLE I
*Comparison of Unstimulated and ConA-Induced Soluble Suppressor Factors Obtained from Various Sources*

| Group | SSF source | No. of patients/group* | % suppression‡ of PFC SSF dilutions 1:3 | P§ | 1:27 | P |
|---|---|---|---|---|---|---|
| 1 | Healthy heterosexual | 14 | 35.9±31.9 | — | +12.4±18.3 | — |
| 2 | Healthy homosexual | 8 | 16.6±37.8 | 0.22 | +0.9±6.1 | 0.32 |
| 3 | Healthy heterosexual, Con A-stimulated | 14 | 92.9±7.4 | <0.001 | 24.0±34.4 | 0.16 |
| 4 | AIDS/hyperplastic lymphadenopathy | 16 | 78.3±30.0 | <0.001 | 59.2±40.7 | <0.0001 |

* For groups 1 and 2, only those supernatants showing ≥20% suppression at a titer of 1:3 were elevated at a 1:27 dilution.

‡ Mean suppression±SD. A positive value represents mean PFC enhancement.

§ *P* indicate differences from group 1 as calculated by *t* test using the transformation: $\sqrt{1-x}$.

*togenic responses to mitogen and antigen.* The viability of cells collected from PWM-driven indicator cultures was determined by trypan blue dye exclusion and $^{51}Cr$-labeled lymphocyte survival. No differences among cultures exposed to supernatants from control or AIDS individuals were noted. Examination of the influence of SSF-AIDS on DNA synthetic responses induced by Con A, PHA, and PWM provided further evidence that SSF is not directly cytotoxic. Supernatants from unstimulated control PBMC and from AIDS-Ton were incapable of altering Con A or PHA-induced T cell transformation, while SSF-Con A blocked this reactivity (Table II). Similarly, [$^3H$]thymidine incorporation in PWM-driven target cultures was unaffected by the presence of SSF-AIDS.

The effect of SSF on antigen-induced T cell proliferation was investigated by addition of supernatants, at a final dilution of 1:5, to indicator cultures prepared with PBMC from individuals previously sensitized in vitro to tetanus toxoid. Seven of seven SSF-AIDS samples which showed a high titer (>1:27) in the PFC assay, but only one of three SSF-AIDS supernatants which showed lower titers, and zero of seven supernatants from healthy heterosexual and homosexual individuals were capable of expressing a clear inhibitory influence (>25% inhibition; Table III). These experiments were repeated with preincubation of $3 \times 10^6$ normal donor PBMC or T cells for 2 h at 37°C in SSF-AIDS and were followed by extensive washing of the cells with phosphate-buffered saline. $2 \times 10^5$ PBMC or $2 \times 10^5$ preincubated T cells plus fresh autologous adherent cells were then cultured with tetanus toxoid for 6 d in culture medium. Approximately 70% inhibition of [$^3H$]thymidine uptake by donor cells preincubated with SSF from AIDS-Car was observed.

TABLE III
*Effect of Lectin-free Supernatants on T Cell Proliferative Responses to Tetanus Toxoid*

| Supernatant source | Percent suppression* |
|---|---|
| 1. Controls | |
| A | 6.6 |
| B | 15.1 |
| C | 17.4 |
| D | 17.9 |
| E | 20.3 |
| F | 23.8 |
| G | 25.3 |
| | mean 18.1 |
| 2. AIDS: SSF titer ≤ 1:27 | |
| H | 21.0 |
| I | 23.3 |
| J | 28.0 |
| | mean 24.1 |
| 3. AIDS: SSF titer ≥ 1:27 | |
| Car | 52.1 |
| Co | 35.4 |
| Ches | 45.8 |
| Gab | 33.9 |
| Knob | 59.4 |
| Lav | 30.9 |
| Sel | 87.4 |
| | mean 49.3 |

These data are pooled from three separate experiments, in which several supernatants were evaluated multiple times and yielded comparable results. Supernatant from a human T-T hybridoma, prepared in our laboratory, which secretes a soluble inhibitory factor, was used as a positive control in these experiments, consistently giving ≥90% suppression.

* Calculated as described in the text.

*Indirect action of SSF-AIDS on B cell function.* To determine whether SSF acts directly on the B lymphocyte to inhibit Ig production, T-independent polyclonal B cell activation was investigated. In contrast to the dependence of the PWM response on helper T cells and monocytes (14), the activation of B cells by EBV is independent of T cells, and less dependent on monocytes (15). SSF from AIDS-Ton did not inhibit and, at a 1:3 dilution, stimulated polyclonal antibody secretion by EBV-induced allogeneic purified B or B plus adherent cells. Supernatant from a control individual had no effect on PFC, while SSF-Con A blocked Ig synthesis (Table IV).

SSF's capacity of affecting primarily the induction phase of Ig formation while permitting an established synthetic response to proceed, was shown by exposing $1.5 \times 10^5$ cells of the IgM-secreting B lymphoblastoid

TABLE II
*Effect of Unstimulated and Con A-activated Supernatants on T Lymphocyte Mitogenesis*

| Experiment | Suupernatant source* | Lectin in indicator culture | Stimulation index‡ |
|---|---|---|---|
| 1 | None | Con A | 22.6±10.2 |
| | $Control_1$ | Con A | 22.6±0.2 |
| | $Control_1$-Con A-activated | Con A | 1.6±0.9 |
| | AIDS-Ton | Con A | 28.9±0.5 |
| 2 | None | PHA | 68.1±3.9 |
| | $Control_2$ | PHA | 61.2±6.7 |
| | AIDS-Ton | PHA | 64.1±3.1 |

* Added in a final concentration of 1:9 vol/vol to indicator cultures.
‡ Mean stimulation index, calculated as described in the text, ±SE.

Table IV
*Effect of Unstimulated and Con A-activated Supernatants on EBV-Stimulated Ig Synthesis*

| Supernatant source | Supernatant dilution | PFC/slide* |
|---|---|---|
| 1. None | — | 201±21 |
| 2. $Control_1$ | 1:3 | 220±40 |
| 3. $Control_1$ | 1:3 | 94±16 |
| Con A-activated | 1:27 | 232±24 |
| 4. AIDS-Ton | 1:3 | 498±22 |
| | 1:27 | 256±12 |

* Each slide represents 1/10 of the contents of an indicator culture microwell, which contains a total of $7.5 \times 10^4$ B cells.

cell line Se D to SSF for varying time intervals (0–24 h). No change in PFC response of these cells was observed.

The above experiments suggest that SSF-AIDS acts via a regulatory cell, presumably a T lymphocyte, monocyte, or dendritic cell. The indirect action of SSF on B cell function was further supported by examining the kinetics of the SSF effect. Subsequent to a 48-h exposure of PBMC to PWM or antigen, the monocyte-dependent phases of the immune response are complete, and the exponential expansion of specifically stimulated clones of Ig-synthesizing cells has begun (16). The effect of delayed addition of SSF-AIDS on PWM-driven PFC is shown in Fig. 2. Introduction of AIDS-Ton SSF at 48 h after culture initiation had no effect on the ultimate PFC response; this suggests that SSF interferes with the orderly differentiation of the B cell to a mature plasmacyte at the level of the helper T lymphocyte or accessory cell.

*Elaboration of SSF by different PBMC subsets.* To establish the cell(s) responsible for SSF production, supernatants from unstimulated, E-rosette$^+$, adherent cell-depleted lymphocytes, PBMC devoid of adherent cells, and adherent cells, were isolated from AIDS-Sel and ID/thy-M.P. Supernatants recovered from 48-h cultures of PBMC or isolated adherent cells (containing 10–15% T lymphocytes) of AIDS-Sel markedly inhibited PFC generation, while purified AIDS-Sel T cell supernatants had no effect (Fig. 3). In contrast, depletion of adherent cells from the PBMC of a patient with ID/thy(M.P.) had no effect on subsequent SSF elaboration, which was of low titer and restricted to the T cell population (Fig. 3).

As monocytes and perhaps other adherent cells are activated by surface adherence (17), and could be secreting SSF as a consequence of such in vitro activation, monocyte preparations were also obtained by Percoll gradient density centrifugation. Supernatants derived from cells found at the 25–40% Percoll interface, which consisted of 85–90% monocytes by indirect immunofluorescence with the monoclonal antibody 63D3, gave PFC inhibition comparable to that attained with SSF derived from plastic adherent cells (data not shown).

To determine whether adherent cells served as the sole source of SSF activity, or if other cells could recruit them to produce the effector molecule in a two-step pathway reminiscent of T or B lymphocyte-macrophage interactions in murine (18–21) and human (22, 23) systems, recombination experiments were performed. Fig. 4 illustrates one of three studies, which utilized supernatants derived from isolated cell subpopulations from two healthy heterosexual controls and a male homosexual with generalized lymphadenopathy and esophageal candidiasis (AIDS-Gab). Combination of $5 \times 10^6$ $control_1$ adherent cells with $2.5 \times 10^6$ $control_2$ T cells in culture medium revealed no inhibition of PWM-driven PFC by supernatants derived at 48 h. Addition of AIDS-Gab T cells to $control_1$ adherent cells

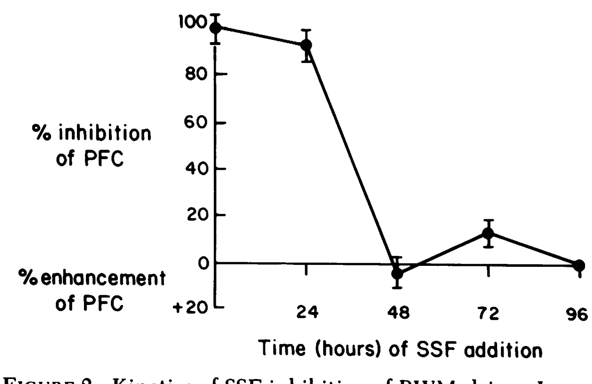


Figure 2 Kinetics of SSF inhibition of PWM-driven Ig synthesis. A 1:1,000 dilution, vol/vol, of AIDS-Ton was added to indicator cultures at the times shown. All cultures were harvested and evaluated for PFC on day 6.

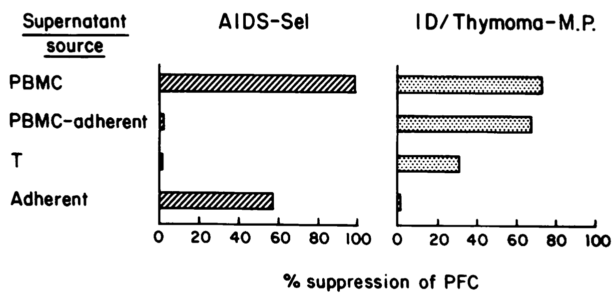


Figure 3 Elaboration of SSF by different PBMC subpopulations: comparison of AIDS and ID/thy. 48-h supernatants were prepared from whole PBMC, PBMC depleted of adherent cells by plastic adherence and carbonyl iron ingestion, and E-rosette$^+$ adherent cell-depleted T lymphocytes. All supernatants were added in a final concentration of 1:3, vol/vol, to indicator cultures.

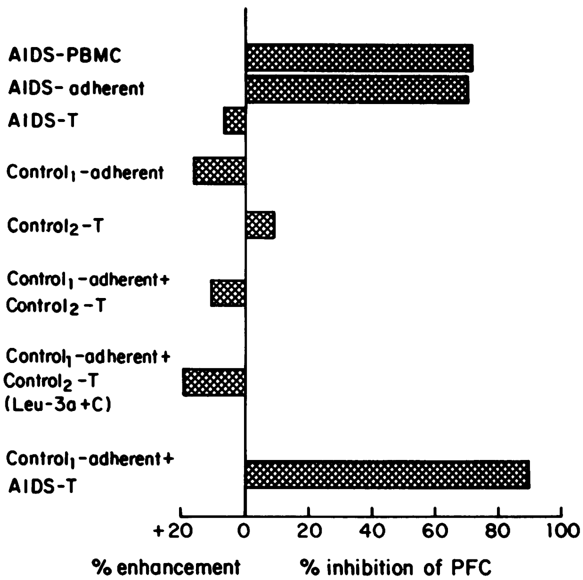


FIGURE 4 Generation of SSF by interaction of AIDS T cells with normal monocytes. Supernatants derived from 48-h cocultures of autologous and allogeneic PBMC subpopulations were added in a final concentration of 1:27 to PWM-driven PFC indicator cultures. $Control_2$-T (Leu-3a + C) indicates pretreatment of T lymphocytes with Leu-3a and complement.

under the same conditions resulted in marked SSF activity.

The apparent collaboration of AIDS T cells with adherent non-T lymphocytes in SSF generation could conceivably be a consequence of the pronounced excess of phenotypic suppressor T cells, which is characteristic of most individuals with AIDS. $Control_2$ T cells were thus treated with Leu-3a and complement. Incubation of $5 \times 10^6$ $control_1$ adherent cells with $2.5 \times 10^6$ Leu-$2a^+$ $control_2$ T cells for 48 h revealed no SSF activity in supernatant dilutions > 1:3 (Fig. 4).

*Effect of prostaglandin inhibitors, anti-interferons, and reducing agents on SSF activity.* Prostaglandins regulate lymphokine secretion, T cell mitogenesis, and Ig production in animal systems, and represent an immunoregulatory product of human suppressor macrophages (24). Alpha and gamma interferons block in vitro Ig synthesis and T cell proliferation to antigen and mitogen (24). Indomethacin in concentrations from 1–10 $\mu$g/ml completely abrogates prostaglandin synthesis by interfering with cyclooxygenation of arachidonic acid (25). Indomethacin utilized in these concentrations, as well as rabbit anti-human gamma interferon and sheep anti-human alpha interferon (1–10 U/culture), had no effect on SSF-induced PFC inhibition when added at culture initiation (data not shown).

The functional relationship of SSF-AIDS to another inhibitory factor, soluble immune response suppressor (SIRS), derived from Con A-treated PBMC in mouse (18) and man (11, 26), was assessed by use of reducing compounds known to mitigate SIRS expression (27, 28). $3 \times 10^{-5}$ M 2-mercaptoethanol (2-ME) was added on culture initiation to PWM-driven indicator cells in the presence or absence of SSF. This concentration of 2-ME was found to have minor effects on PFC in the absence of cell supernatant and yet, to be sufficient to overcome SSF-Con A-induced PFC suppression (data not shown). As seen in Table V, addition of 2-ME along with various dilutions of SSF-AIDS resulted in blockade of PFC inhibition, with complete abolition of SSF activity at supernatant dilutions > 1:6. Ascorbic acid was less effective; a $1 \times 10^{-4}$ M concentration was capable of blocking the effect of SSF-AIDS used in the experiment of Table V by only 50%. Increasing the concentration of the ascorbic acid further resulted in toxicity, with suppression of PFC in the absence of SSF.

## DISCUSSION

PBMC from AIDS patients or homosexual males with unexplained hyperplastic lymphadenopathy, which were cultured for 48 h in the absence of mitogenic stimulation, elaborated SSF capable of inhibiting helper T cell-dependent immune reactivity. SSF was unable to block T-independent processes, including polyclonal Ig production by EBV-stimulated B lymphocytes. Monocyte responses, represented by peroxide activity and bactericidal function (R. Snyderman and H. Murray, personal communication), and natural killer cell cytotoxicity directed against K562 targets were similarly unaffected by SSF. Genetic restriction of SSF was not observed in experiments that compared the activity of

TABLE V
*Effect of 2-ME on SSF Inhibition of PWM-driven Ig Synthesis**

| SSF source | SSF dilution | 2-ME | PFC/slide | Inhibition of PFC |
|---|---|---|---|---|
| | | | | % |
| None | — | — | 171±21 | — |
| AIDS-Knob | 1:6 | — | 9±9 | 94.7 |
| AIDS-Knob | 1:12 | — | 10±6 | 94.2 |
| AIDS-Knob | 1:24 | — | 12±3 | 93.0 |
| None | — | + | 113±20 | — |
| AIDS-Knob | 1:6 | + | 57±3 | 49.6 |
| AIDS-Knob | 1:12 | + | 151±17 | 0 |
| AIDS-Knob | 1:24 | + | 160±6 | 0 |

* $3 \times 10^{-5}$ M 2-ME was added on initiation of indicator cultures along with the requisite dilutions of supernatant from cultures of the PBMC of a male homosexual with generalized hyperplastic lymphadenopathy (AIDS-Knob).

supernatants derived from control and AIDS patients with HLA-DR allotypes identical to or divergent from cells in PFC indicator cultures.

The exact mechanism of SSF effector function is unclear. If SSF directly inhibits T cells, then not all such cells share equivalent susceptibility. The amount of SSF required to depress PWM-driven polyclonal Ig formation (Fig. 1, Table I) is much lower than that necessary to diminish T cell proliferation responses to specific antigen (Table III). A similar dichotomy in dose-response was described for Con A-derived soluble factors (11, 26). In contrast to inhibition of tetanus toxoid-induced blastogenesis, DNA synthetic responses to optimal mitogenic concentrations of Con A, PHA, and PWM were uniformly unaffected by SSF-AIDS (Table II). This may relate to the disparate requirements for monocytes (14, 19), dendritic cells (30), or lymphokines among the T-dependent indicator assays utilized. As SSF must be present during the initial 24–48 h of the PWM-driven PFC system, the period during which maximal helper factor activity is observed (31), interference with lymphokine synthesis, secretion, or receptor capacity may also play a pivotal role in the SSF effect. The existence of such pathways for suppressor T cells and their factors in murine models is well recognized (21, 32).

SSF-AIDS-mediated inhibition is partially reversible by high concentrations of 2-ME. This effect is of special interest as it implicates mechanisms analogous to the Con A-derived SIRS. SIRS is a 55,000 D glycoprotein product of Con A-activated T cells which blocks primary and secondary IgM and IgG responses in vitro, DNA synthetic reactions to T and B cell mitogens, blastogenesis by several tumor cell lines (18), as well as in vivo immune reactivity (33). It is made by murine $Ly2^+$ T cells in an inert precursor form and is converted into the active moiety upon oxidation by $H_2O_2$ derived from stimulated monocytes (18). SIRS-mediated suppression of PFC responses to antigen is circumvented by reagents that prevent formation of the macrophage-modified soluble factor (catalase and cyanide), or inactivate it (reducing agents, including ascorbic acid, amines, and sulfhydryl compounds such as 2-ME [27]). These data suggest that the effector function of SIRS involves oxidation of cellular components that are essential for mitosis. SSF-AIDS shares several characteristics with SIRS, including time course of generation, kinetics of PFC inhibition, serum dependency, absence of cytotoxicity, sensitivity to reducing agents, and the T cell-adherent cell interaction needed for its production. It differs from SIRS in two key respects, however. The primary function abrogated by the Con A-induced product is cell division, while the SSF-AIDS effect is independent of such proliferation. Furthermore, murine and human Con A factors inhibit EBV-induced Ig production in the absence of T cells. It is possible that Con A induces a multiplicity of factors, one of which relates closely to SSF-AIDS.

It should be feasible to determine if the SIRS-macrophage network is involved in other immunoregulatory systems associated with soluble products, including SSF elaborated in low titer by lectin-free cells from some healthy control individuals (Fig. 1, Table I), by examining the ability of compounds that inactivate SIRS to block their inhibitory capacity. By this criterion, suppression of PFC responses by viral (type I) interferon is mediated at least in part by the SIRS-macrophage pathway (18). Interferons are unlikely candidates for regulating SSF-AIDS activity, however, as heteroantisera to alpha and gamma interferons had no effect on SSF-treated target cultures. In addition to the cases of viral and bacterial infection and multiple myeloma described here, SSF revealed PFC inhibition in excess of control levels in a subset of patients with systemic lupus erythematosus and severe combined immune deficiency, but only at a 1:3 dilution. Evidence for SSF-AIDS activity in supernatants prepared from PBMC obtained from patients during or directly after episodes of opportunistic infection was found in only 1 of 10 samples. One hyperplastic lymphadenopathy patient no longer had detectable levels of SSF when retested after development of *Pneumocystis carinii* pneumonia. This raises the possibility that those cells responsible for generation of SSF-AIDS may be altered during periods of active disease, typically characterized by marked leukopenia.

Simple demonstration of cell or soluble factor-mediated inhibitor effects in vitro does not, of course, establish their significance in the etiology of the disorder in which they are found. A prospective follow-up of the male homosexuals examined in this study, as well as of persons in other risk groups for AIDS who presented high titers of SSF, is mandatory to establish the clinical importance of this substance. For example, a soluble macrophage factor capable of inducing suppressor T cells was identified in patients with disseminated fungemia (22). Persistence of inhibitory influences over a 2–3-yr period was recorded during disease-free intervals in a subset of patients; only these individuals, and not those lacking soluble macrophage factor, proved to be at increased risk for mycotic relapse (34).

This in vitro system provides an accessible model with which to examine cell subpopulations involved in the activation and expression of immunoregulatory defects in AIDS and in other conditions associated with immune dysregulation. It may also assist in establishing leads to agents of potential therapeutic efficacy.

*Note added in proof.* The 16 AIDS/hyperplastic lymphadenopathy patients utilized in this study have been followed for a mean of 14 mo (range 7–21 mo). Two of the four AIDS patients have expired from opportunistic infections. Of the 12 homosexual males with lymphadenopathy and systemic symptoms, 4 have stable signs and symptoms, 3 have evidenced resolution of clinical findings, and 5 have developed AIDS, including 2 deaths. Of the two lymphadenopathy patients with SSF titers of 1:1,000, AIDS-Ton and AIDS-Sel, the former developed Pneumocystis pneumonia and died within 11 mo of follow-up, while the latter has had a reduction in peripheral adenopathy, resolution of low grade fevers and malaise, and disappearance of SSF.

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